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Differences in spectral sensitivity within and among species of darters (genus *Etheostoma*)

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ABSTRACT

We examined variation in the visual system both within and among seven species of darters, colorful freshwater fishes of the genus *Etheostoma*. Using microspectrophotometry, we found that darters possess rod photoreceptor cells, single cone photoreceptor cells containing middle wavelength sensitive (MWS) visual pigments, and twin photoreceptor cells containing (LWS) visual pigments. No variation in peak sensitivity was detected among species or individuals in the rod class. In the MWS class, significant variation was detected among species and a strong statistical trend suggests differences among individuals. By contrast, all differences in the LWS class could be attributed to variation among individuals. Patterns of variation detected among species, among individuals, and among cone classes suggest that complex patterns of selection may be shaping the visual system of these fishes. Further, differences among individuals may have important consequences for visually based behaviors.

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1. Introduction

Differences in visual system characteristics within and among species may reflect important differences in ecology and behavior, as visual systems are shaped by natural selection to optimize the efficiency of visually mediated tasks in local light environments (Carleton et al., 2005; Endler, 1993; Seehausen et al., 2008). Aquatic light environments are especially variable (Kirk, 1983) and behavioral variation is related to differences in spectral qualities due to depth (Cummings, 2007; Seehausen et al., 2008), organic particulates (Boughman, 2001; Fuller, Houle, & Travis, 2005), and filtering properties of surrounding vegetation (Endler, 1995). Selective pressures in various habitats can have important implications for communication among individuals (Dalton, Cronin, Marshall, & Carleton, 2010) and, ultimately, can play a role in speciation. For example, fish systems provide ample evidence that the adaptation of visual systems to their environments can affect the process of sexual selection. In a number of freshwater fish species, visual courtship signals, typically breeding coloration in males, have been shown to evolve in response to natural selection on visual systems (Boughman, 2001; Cummings, 2007; Fuller, Houle, & Travis, 2005; Seehausen et al., 2008).

While species and population level differences in visual sensitivity are well studied, variation in spectral sensitivity among individuals within a population is less often quantified (Bowmaker, Loew, & Liebman, 1975). Individual variation has been examined in fishes,

and studies are beginning to examine the underlying causes and potential consequences of individual variation in vision in these systems. In sand gobies (*Pomatoschistus minutus*), populations differ in the peak absorbance of rods, and one population in particular exhibits a wide range of absorbance values (Jokela-Maatta et al., 2009). This is due to some individuals having nucleotide substitutions in the rod opsin sequence causing a red shift in the absorbance peak (Jokela-Maatta et al., 2009). In guppies (*Poecilia reticulata*), microspectrophotometric studies showed multiple absorption peaks in the long-wavelength sensitive photoreceptor class, with individuals expressing different combinations of absorption peaks (Archer & Lythgoe, 1990). Molecular analysis further supports that guppies vary in spectral sensitivity at the population and individual level, with individuals expressing different numbers of long wavelength sensitive opsins, up to four in one individual (Hoffmann et al., 2007).

We aimed to examine variation in the visual system both within and among species of darters, freshwater fishes of which breeding males are particularly colorful (Fig. 1). There are approximately 201 species of darters representing four recognized genera, of which *Etheostoma* is the largest genus of North American freshwater fishes (Page & Burr, 2011). Although males of most *Etheostoma* species are characterized by elaborate, species-specific nuptial color patterns, the visual systems of darters have not yet been described. As in other systems, darter visual systems may be tuned to maximize efficiency for visually mediated tasks in particular environments. Darter species are found in a variety of macrohabitats, from small streams up to medium rivers, and microhabitat preferences vary among some species (Boschung & Mayden, 2004; Etnier

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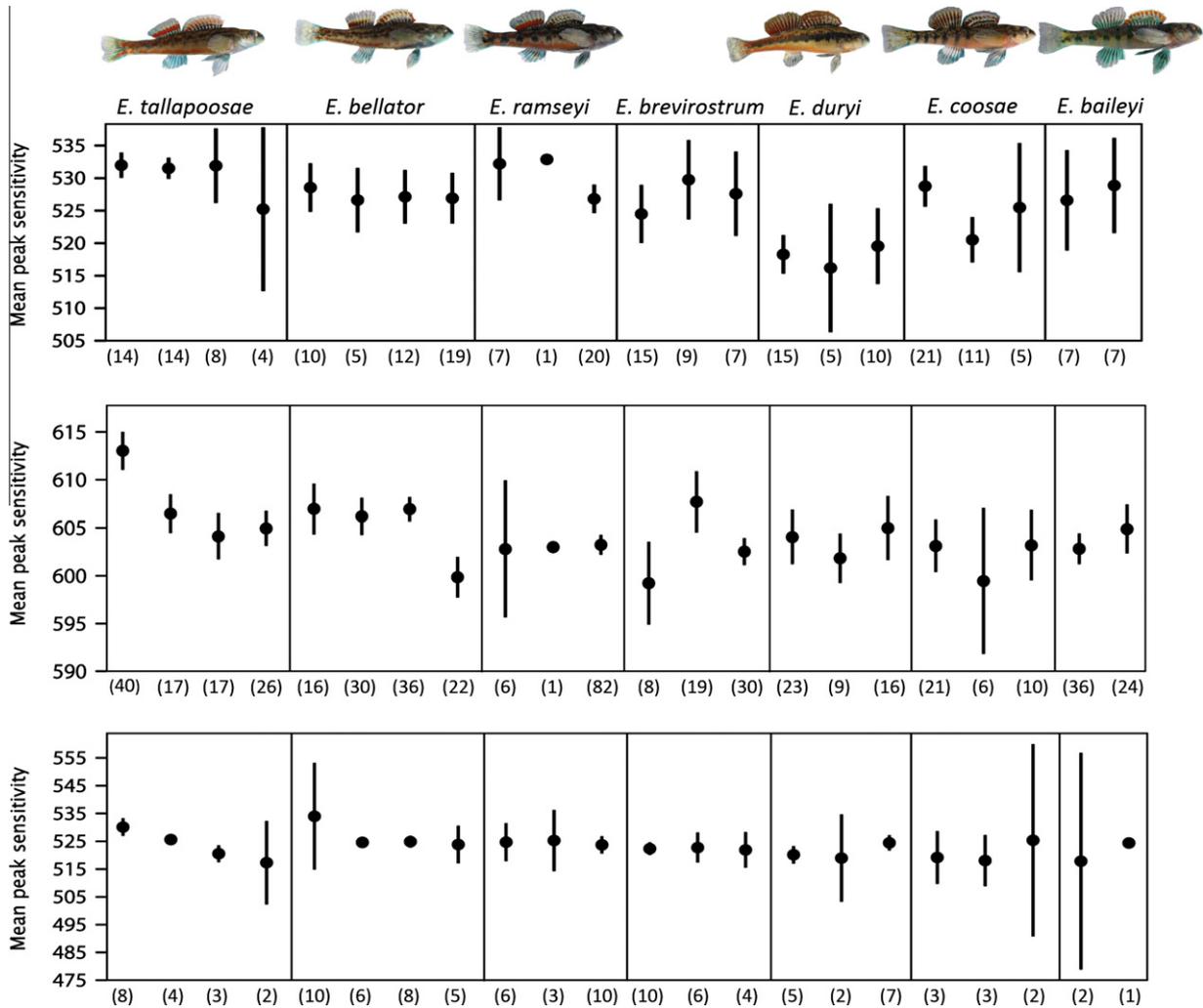 *E-mail address:* jgumm@umbc.edu (J.M. Gumm).

Fig. 1. Mean and 95% confidence interval of peak wavelength of spectral sensitivity for individual darters. (A) *E. tallapoosae*, (B) *E. bellator*, (C) *E. ramseyi*, (D) *E. brevirostrum*, (E) *E. duryi*, (F) *E. coosae*, (G) *E. baileyi*. Males of most species are shown at the top of each corresponding column. The top panel represents the MWS cone class, the middle panel the LWS cone class and the bottom panel the rod class. Numbers in parentheses represent the sample size of photoreceptors sampled for that individual.

& Starnes, 1993). Further, vision appears to be directly involved in divergence and speciation in this group. Females have mate preferences based on visual cues (Williams & Mendelson, 2010, 2011; Martin & Mendelson, unpublished data) and sexual, or behavioral, isolation evolves faster than hybrid inviability; that is, the first reproductive barrier to arise between diverging lineages is the failure to recognize each other as suitable mates (Mendelson, 2003; Mendelson, Imhoff, & Venditti, 2007).

We examined visual pigment absorbance in the subgenus *Ulocentra* of the genus *Etheostoma*. Fishes in this subgenus are commonly known as snubnose darters, and the subgenus contains approximately 19 species (taxonomic revisions ongoing) (Bailey & Etnier, 1988; Mendelson & Wong, 2010; Porter, Cavender, & Fuerst, 2002; Smith, Mendelson, & Page, 2011). Male snubnose darters show especially elaborate nuptial coloration on the body and fins during the breeding season, March through May, and the colors are used in sexual communication during competitive interactions with males and courtship towards females. Patterns of nuptial coloration are often used as diagnostic characters to distinguish closely related species of *Ulocentra* (e.g., Page & Burr, 2011; Porter, Cavender, & Fuerst, 2002; Powers & Mayden, 2003). Finally, studies of the evolutionary patterns of male nuptial coloration in this subgenus suggest that multiple selection pressures are acting on coloration, and these selective mechanisms may also involve

the evolution of divergent visual systems (Gumm & Mendelson, 2011).

2. Materials and methods

2.1. Collection and maintenance of fishes

We measured outer segment absorbance from rods and cones of females ($N = 2–4$) of seven species of snubnose darters (genus *Etheostoma*, subgenus *Ulocentra*). Females were used for the study due to established relationships between female vision and male nuptial coloration in other freshwater fish species. Fishes were collected by seine net in March and April, 2010 (Table 1). Fishes were transported by car in aerated coolers to the University of Maryland, Baltimore County where they were held for up to 4 weeks. In the lab, fishes were separated by species and were housed in 10-l tanks on a re-circulating aquarium system (Aquatic Habitats, Inc.). Fishes were maintained at a constant temperature of 12–14 °C on a 12:12 L:D cycle consisting of fluorescent bulbs in addition to natural lighting through standard glass windows. Fishes were fed live blackworms daily until they were transported by car in aerated coolers to Cornell University, where they were analyzed in May 2010.

Table 1
Darter species sampled, collection location, number of fish sampled and peak absorbance for middle wavelength and long wavelength photoreceptors. Numbers in parentheses are the number of photoreceptors sampled for each class and species.

Taxon	Collection location	Sample size	Rod Mean ± s.d.	MWS Mean ± s.d.	LWS Mean ± s.d.
E. baileyi	Middle Fork of the Red River, Powell Co., KY	N = 2	520.01 ± 4.87 (3)	527.74 ± 7.89 (14)	603.64 ± 5.31 (60)
E. bellator	Blue Spring Creek, Blount Co., AL	N = 4	524.85 ± 3.09 (29)	527.3 ± 6.61 (46)	605.26 ± 5.32 (104)
E. brevirostrum	Shoal Creek, Cleburne Co., AL	N = 3	522.38 ± 3.76 (20)	526.72 ± 7.88 (31)	603.79 ± 5.8 (57)
E. coosae	Shoal Creek, Cleburne Co., AL	N = 3	520.3 ± 4.5 (8)	525.8 ± 7.45 (37)	602.37 ± 5.8 (37)
E. duryi	Sweetens Creek, Marion Co., TN	N = 3	522.12 ± 3.5 (14)	518.34 ± 6.65 (30)	603.94 ± 5.97 (48)
E. ramseyi	Schultz Creek, Bibb Co., AL	N = 3	524.25 ± 4.87 (19)	528.39 ± 5.45 (28)	603.21 ± 4.8 (89)
E. tallapoosae	Buck Creek, Clay Co., AL	N = 4	525.86 ± 5.50 (17)	531.13 ± 4.85 (40)	608.29 ± 6.45 (100)

2.2. Microspectrophotometry (MSP)

Fishes were dark adapted for at least 12 h before MSP measurements were performed. Under dim red light, fishes were anesthetized with MS-222 and euthanized by cervical dislocation. Using an image converter, eyes were enucleated and hemisected under infrared light. Whole retinas were separated from the pigment epithelium and mounted on a coverslip in phosphate buffer (pH 7.2) containing 6% sucrose. They were cut into small sections and teased apart with scalpel blades and sealed under a second slip with vacuum grease. While no systematic measurements from different retinal areas were made, the retinal preparation tended to randomize the sample in regards to sectors and central vs. peripheral retina.

Absorbance spectra were recorded from all identifiable cone and rod cells with a single beam microspectrophotometer, the details of which have been previously described (Carleton et al., 2005; Loew, 1994). Briefly, after acquisition of a baseline spectrum from a clear area of the preparation, an individual photoreceptor was located under infrared illumination and placed over the measuring beam. Data were obtained at one nanometer interval scans from 750 nm to 350 nm and back to 750 nm. Absorbance spectra were analyzed in Excel to assess the goodness of fit of the spectra to standard 'Govardovskii' templates (Govardovskii et al., 2000). Templates with pure A1, pure A2 and A1/A2 chromophore mixtures were assessed for fit to the data and the lambda max, or peak absorbance, was determined from the best-fitting template. Only those data meeting the selection criteria (e.g., appropriate alphaband shape, lack of significant short-wavelength scatter, etc.) given in Loew (1994) were included in the final analysis.

2.3. Statistical analyses

The resulting values of lambda max for each cell type were normally distributed and met all assumptions of parametric statistics. We tested for differences among species using analysis of variance. Given that multiple photoreceptors were measured from each individual, we used a nested design with individual identity as a random factor nested within the factor accounting for species. Tukey's HSD post hoc tests were used to determine which species or individuals differed.

3. Results

3.1. Cell types and visual pigments

The retinas of Etheostoma possessed rod photoreceptors and both single and double cones. Lambda max values for rod cells ranged between 513 and 535 nm and species means ranged from 520 to 525 nm. The double cones were twin cones, with the outer segments of each member being morphologically similar in size and shape. Two classes of visual pigments were characterized from

cone cells and are classified as a middle-wavelength sensitive class (MWS) and a long-wavelength sensitive class (LWS). Double cones always contained LWS visual pigments and single cones contained MWS visual pigments. Cones in the MWS class had lambda max values between 504 and 543 nm and cones in the LWS class had lambda max between 582 and 628 nm. Species means ranged from 518 to 531 nm for the MWS class and 602 to 608 nm for the LWS class (Table 1). In almost all samples, a pure A2 chromophore template provided the best fit to the data.

3.2. Variation among and within species

For rod photoreceptors, there were no significant differences among species (Nested ANOVA: $F_{(6,88)}$ = 0.961, P = 0.472) or individuals (Nested ANOVA: $F_{(15,88)}$ = 0.891, P = 0.576) in peak sensitivity (Fig. 1). For cone photoreceptors in the LWS class, there were no differences in peak sensitivity among species (Nested ANOVA: $F_{(6,473)}$ = 0.903, P = 0.517), but there were significant differences among individuals (Nested ANOVA: $F_{(15,473)}$ = 7.688, P < 0.001; Fig. 1). Finally, for cone photoreceptors in the MWS class, there were significant differences in peak sensitivity among species (Nested ANOVA: $F_{(6,204)}$ = 6.260, P = 0.001) and a non-significant trend for differences among individuals (Nested ANOVA: $F_{(15,204)}$ = 1.689, P = 0.056; Fig. 1).

4. Discussion

4.1. Cone types in darters

This study is the first to report the spectral sensitivity of darters. Fishes from the subgenus Ulocentra possess rod photoreceptor cells, single cone photoreceptor cells containing MWS visual pigments, and twin photoreceptor cells containing LWS visual pigments. Despite extensive sampling, our measurements did not reveal any cone cells containing short wavelength sensitive or UV sensitive pigments, although these could be present and may have remained undetected due to occurrence at a very low frequency or uneven distribution in the retina (Jordan et al., 2006; Parry et al., 2005). Adult female darters therefore appear to lack SWS and UV sensitivity despite the availability of UV light in the shallow environments where the fishes live and spawn (Gumm & Mendelson, unpublished data) and the prevalence of UV and blue or cyan reflectance in male nuptial coloration (Gumm & Mendelson, unpublished data; Gumm, Feller, & Mendelson, 2011; Gumm & Mendelson, 2011). The most closely related species to have its visual system described is the yellow perch (Perca flavescens). Adult yellow perch also lack SWS or UV cones; however, juveniles possess small, single cones with an absorbance peak around 400 nm (Loew & Wahl, 1991). Ontogenetic changes in yellow perch visual systems are associated with changes in diet and habitat use from pelagic to demersal habitats (Wahl et al., 1993). Darter larvae are

also pelagic (Page & Burr, 2011), thus SWS or UV cones may be present at early life stages and lost at maturity.

4.2. Variation among species and individuals

Patterns of variation in peak photoreceptor sensitivity among species and individuals differed among the photoreceptor classes. Specifically, no variation in peak sensitivity was detected among species or individuals in the rod class. In the MWS class, significant variation was detected among species and a strong statistical trend suggests differences among individuals. By contrast, all differences in the LWS class could be attributed to variation among individuals. These data suggest that patterns of selection may differ on different aspects of the visual system. Rod photoreceptors are used for scotopic (low light) vision, whereas cone photoreceptors are used for photopic, color vision, two distinct visual channels that can respond independently to distinct selective pressures. In the sand goby (*P. minutus*), the strength of selection on the rod visual pigment gene (rhodopsin) has been found to vary among populations. The rhodopsin gene in a population with a stable light environment expresses characteristics consistent with diversifying selection as each population adapts to its unique environment, whereas the same gene sampled from fish in a population with a fluctuating light environment expresses high levels of polymorphism consistent with balancing selection (Larmuseau et al., 2010). Though these results demonstrate variation in patterns of selection on one opsin class, a similar approach may be used to examine variation in patterns of selection on different opsin genes, and across photoreceptor classes (Spady et al., 2005).

In the MWS class, peak absorbance was lower in *Etheostoma duryi* than in any other species (Fig. 1). This difference in the visual system corresponds with differences among species in nuptial coloration. A recent comparative analysis of coloration among snubnose darters found that the spectral location, or hue, of the orange body coloration in *E. duryi* corresponded to a shorter wavelength than in other species analyzed; body color spectral location occurred at wavelengths ranging an average of 3–17 nm longer in other species (Gumm & Mendelson, 2011).

Variation among individuals accounted for differences in peak absorbance of the LWS photoreceptors. Two of seven species examined had significant differences among individuals. In both *Etheostoma tallapoosae* and *Etheostoma bellator*, one of four individuals had lambda max values that differed from the others of that species. Specifically, one individual of *E. tallapoosae* had peak sensitivity at a longer wavelength than all others, and one individual of *E. bellator* had peak sensitivity at a shorter wavelength than all others. The distribution of peak wavelengths of LWS photoreceptors within individuals suggests that multiple alleles may contribute to the patterns of variation seen among individuals. However, small within individual sample sizes and noise in MSP measurements makes this result difficult to interpret clearly. Multiple genetic mechanisms of spectral tuning could explain this variation in visual sensitivity. Absorbance of the visual pigment (e.g., lambda max) is determined by both the opsin protein and the bound chromophore (Bowmaker, 1995). Therefore, changes in the amino acid sequence that occur in the binding pocket portion of the protein may shift visual sensitivity, and specific amino acid substitutions are known to shift sensitivity between 2 and 35 nm (Yokoyama, 2000). A second mechanism of spectral tuning is differential gene expression, which alters spectral sensitivity through regulation of opsin genes. Gene expression differences can essentially turn different suites of genes on and off (Carleton, Harosi, & Kocher, 2000; Carleton & Kocher, 2001; Parry et al., 2005; reviewed in Carleton, 2009) or can vary the degree of expression differences among opsin genes (Fuller et al., 2004). Additional studies are required to

understand how these two mechanisms contribute to the differences within and among species observed here.

Alternatively, peak absorbance may differ based on the identity of the chromophore that is bound to the visual pigment. In fishes, the chromophore may be derived from either vitamin A1 or A2, and retinas may be comprised exclusively of either type or may be a mixture of the two. Chromophore mixing may cause shifts of 5–60 nm in visual pigment absorbance depending on the opsin gene (Hárosi, 1994). Our qualitative analysis of template shape suggests that retinas in darters contain only A2 chromophore. Additionally, if chromophore mixing contributes to variation in peak absorbance, this variation is predicted to be consistent across all photoreceptor types (Loew & Dartnall, 1976) as contiguous cells expressing different chromophores are rare and are not known to occur in Percid fishes (Loew, pers. obs.). Cone photoreceptors varied in our study but rod photoreceptors did not vary, further supporting that chromophore mixing does not account for variation in spectral sensitivity in darters. However, these lines of evidence do not definitively rule out chromophore mixing as a contributing factor to the differences we see among individuals and/or species and future studies should regenerate the pigments *in situ* using either 11-cis or 9-cis retinal to characterize the chromophore makeup of darter retinas (as in Parry & Bowmaker, 2000).

Finally, differences within and among species could be due to regionalisation of the retina. If different photoreceptors or cone photoreceptors expressing different opsins are found in different locations across the retina (Reckel & Melzer, 2003), variation in spectral sensitivity may be accounted for by inconsistent sampling of retinal areas. While no systematic measurements from different retinal areas were made in this study, the methods used to prepare whole retinas tended to randomize the preparation in regards sectors and central vs. peripheral retina.

Both of the species showing variation among individuals represented the largest sample sizes in our study. Sample sizes here ($N = 2$–4) are within the range of those typically reported for MSP analyses of visual systems (Carleton, Harosi, & Kocher, 2000; Jordan et al., 2006). Thus, the significant variation among individuals found here is striking, as individual variation is not typically reported. Variation among individuals may be more prevalent than measured in this study, motivating the need to sample additional individuals of the species included here, and others, to more fully examine patterns of variation in visual systems.

4.3. Conclusions

Sensory systems are compelling examples of how empirical studies can link multiple levels of organization in a single system. Examining the visual system in particular can demonstrate how changes at the genetic level can influence physiology, how physiology can influence individual behavior and, ultimately, how this may influence speciation (e.g., Dangles et al., 2009). This study is the first step towards understanding the extent of variation in the visual systems of darters, a diverse clade of colorful fishes. Patterns of variation detected among species, among individuals, and among cone classes suggest that complex patterns of selection may be shaping the visual system of these fishes. These data provide the foundation for future work on the proximate basis and functional consequences of this variation.

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